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**A comparative study on the *in vitro* effects of the DNA methyltransferase inhibitor
5-Azacytidine (5-AzaC) in breast/mammary cancer of different mammalian species.**

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A comparative study on the *in vitro* effects of the DNA methyltransferase inhibitor 5-Azacytidine (5-AzaC) in breast/mammary cancer of different mammalian species.

Abstract

Murine models are indispensable for the study of human breast cancer, but they have limitations: tumors arising spontaneously in humans must be induced in mice, and long-term follow up is limited by the short life span of rodents. In contrast, dogs and cats develop mammary tumors spontaneously and are relatively long-lived. This study examines the effects of the DNA methyltransferase (DNMT) inhibitor 5-Azacytidine (5-AzaC) on normal and tumoral mammary cell lines derived from dogs, cats and humans, as proof of concept that small companion animals are useful models of human breast cancer. Our findings show that treatment with 5-AzaC reduces *in vitro* tumorigenicity in all three species based on growth and invasion assays, mitochondrial activity and susceptibility to apoptosis. Interestingly, we found that the effects of 5-AzaC on gene expression varied not only between the different species but also between different tumoral cell lines within the same species, and confirmed the correlation between loss of methylation in a specific gene promoter region and increased expression of the associated gene using bisulfite sequencing. In addition, treatment with a high dose of 5-AzaC was toxic to tumoral, but not healthy, mammary cell lines from all species, indicating this drug has therapeutic potential. Importantly, we confirmed these results in primary malignant cells isolated from canine and feline adenocarcinomas. The similarities observed between the three species suggest dogs and cats can be useful models for the study of human breast cancer and the pre-clinical evaluation of novel therapeutics.

Keywords

5-Azacytidine (5-AzaC), mammary cancer cell line, primary mammary tumor, dog, cat, human

Background

Murine cancer models such as genetically engineered mice and human-mouse xenograft models have been extremely useful in studies on the complexity of human breast cancer, and these models are generally accepted to be the most effective means for studying and understanding breast cancer development [1]. However, and despite the unquestionable importance of these murine models in human cancer research, they have some limitations. The most significant is that tumors arise spontaneously in humans, but have to be induced in most mouse models. Furthermore, biological differences between naturally occurring cancers in humans and transplanted cancers in mice can lead to divergences in carcinogenesis including differences in telomerase activity, variation in activated gene sets and pathways, and changes in tolerance to certain drugs and proteins [2]. In addition, tumor development and responses observed in mouse models are not always predictive of human tumors of similar histology, and long-term follow up studies on tumor growth are limited due to the relatively short life span of mice. Therefore, more appropriate, spontaneous animal models that fully recapitulate the complex biology of breast cancer in human patients are needed. Spontaneous tumors in dogs and cats share many features with their human counterparts and offer valuable supplementary model systems for research aimed at elucidating important molecular mechanisms and gene signaling pathways that have a role in mammary tumorigenesis [3-5]. In addition,

mammary cancer in dogs and cats is an underused, but unique resource for preclinical, translational research on cancer therapeutics because the hepatic enzyme homology of these animals is much more similar to humans than that of rodents, allowing for a more accurate extrapolation of pharmacokinetics, toxicity and dosing of anti-cancer drugs for both human as well as animal use [3, 6, 7].

Breast cancer is induced by the accumulation of altered gene regulation. Besides abnormalities in the DNA sequence (genetic mutations), changes in gene expression profiles (epigenetic dysregulation) also have an important role in breast cancer tumorigenesis [8, 9]. Because of the reversible nature of epigenetic alterations, the potential of epigenetic modifiers in breast cancer drug development has gained significant interest [10]. Several drugs that target epigenetic alterations, including inhibitors of histone deacetylase (HDAC) and DNA methyltransferase (DNMT), are currently approved for treatment of hematological malignancies and are being explored for clinical investigation in solid tumors, like breast cancer [8, 10-12]. For example, the DNA methyltransferase inhibitor 5-AzaC is a Food and Drug Administration (FDA) approved drug used clinically to treat acute myelodysplastic leukemia [13] and has been shown to inhibit cell invasiveness and growth of several human breast cancer cell lines [14,15].

In contrast, much less is known about gene signatures in canine, and especially feline, mammary cancer although this is imperative information to further strengthen the value of naturally occurring mammary cancer in these animals as a model for human breast cancer. Moreover, the potential of epigenetic modifiers, like 5-AzaC, as anti-cancer drugs to treat mammary cancer in these species has only minimally been explored.

Therefore, the aims of the present study were to evaluate the effects of 5-AzaC on mammary tumorigenicity of canine and feline mammary cancer cells *in vitro*, and to compare these results with results obtained in human breast cancer cell lines, in order to further emphasize the importance of dogs and cats as powerful models in which to study human breast cancer as well as explore new treatment options for all three species.

Methods

Established mammary cell lines

The human normal breast epithelial cell line MCF10A and its derivative MCF10CA1a, a poorly differentiated invasive malignant carcinoma cell line, have been intensively characterized [16-19]. MCF10A cells were cultured in Dulbecco's modified Eagle medium (DMEM)/F12 (Corning, Acton, MA) supplemented with 5% horse serum (Gibco), 1% penicillin/streptomycin (Invitrogen), 10 µg/ml human insulin 20 ng/ml epidermal growth factor (EGF) and 0.5 µg/ml hydrocortisone (all from Sigma, St Louis, MO) (Medium A); MCF10CA1a were cultured in DMEM/F12 supplemented with 5% horse serum and 1% penicillin/streptomycin (Medium B). The non-invasive, oestrogen-receptor (ER) positive MCF7 cell line [20,21] was cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin/streptomycin (Medium C).

The feline normal mammary epithelial cell line (FMEC), a kind gift from Dr. John Parker (Baker Institute for Animal Health, Cornell University, Ithaca, NY) [22], the mammary adenocarcinoma cell line K12-72.1, a kind gift from William Hardey Jr. (School of Veterinary Medicine, Cornell University, Ithaca, NY) [23] and the mammary carcinoma cell line CAT-MT [24] were cultured in Medium C. The canine normal

mammary epithelial cell line (CMEC), which was established in-house using mammary gland tissue from a healthy 2-year old female research Beagle and used at passages 40-45, and the mammary carcinoma cell lines REM134 and CMT12 [25, 26] were also cultured in Medium C.

All cell lines were maintained at 37°C in a humidified environment with 5% CO₂.

Primary mammary tumor cell cultures

Tissue samples were obtained from a dog and cat with owner consent for tissue donation. The dog was an 11-year old spayed mixed breed with mammary adenocarcinomas in two glands, but no observed metastasis based on chest radiographs. The cat was a 10-year old unspayed female with palpable mammary adenocarcinomas in three glands, one of which was accompanied by skin ulceration. Tissue samples were collected during surgery (dog sample) or after euthanasia (cat sample), placed in PBS and shipped to the laboratory overnight at 4°C. Tissues were minced into 1- 2-mm³ pieces and digested with 0.1% collagenase type III (Worthington Biochemical, Lakewood, NJ) for 60 min at 37°C. Cell suspensions were subsequently filtered through a sterile 100 and 40 µm filter to obtain a single cell suspension, and centrifuged at 400 g for 10 min at room temperature (RT). Cells were incubated in Medium C at 37°C in a humidified environment with 5% CO₂. In addition, part of the tissue was fixed in 10% neutral buffered formalin and histology sections were send to Cornell Anatomic Pathology for tumor grading.

Anchorage-independent growth assays

Cells for soft agar assays were pretreated for 2 days with 5 µM 5-Azacytidine (5-AzaC), or cultured under standard conditions, after which cells were detached using

(0.25%) Trypsin-EDTA (Corning Life Sciences, Manassas, VA) and counted. To set up soft agar assays, 2 mL of 0.6% 2-hydroxyethylagarose melted in appropriate culture medium were pipetted into wells of 6-well culture plates and plates were held at 4°C for 15 min until the agarose solidified. Ten thousand cells per well were gently resuspended in 1.5 mL 0.3% 2-hydroxyethylagarose melted in appropriate culture medium, and layered over the base agarose. Cells were cultured in soft agar for 7 to 14 days at 37°C with 5% CO₂. Every 3 days, cultures were fed with 1 mL 0.3% 2-hydroxyethylagarose melted in appropriate culture medium. Cultures were photographed at 10x using a Nikon Diaphot-TMD inverted light microscope with an attached Cohu CCD camera (Nikon, Melville, NY). The number of spheres, defined as clusters of cells increasing in size due to cell division, a universal feature of tumoral cell lines [31-34], was counted and average sphere areas were determined using *Image J* software (<http://rsb.info.nih.gov/ij/>).

Electric Cell-substrate Impedance Sensing

Cell proliferation rates were measured by Electric Cell-substrate Impedance Sensing (ECIS) using the ECIS Model Z instrument with 96W array station (ECIS, Applied BioPhysics Inc., Troy, NY). To this end, cells were pre-treated for 48 h with 5 µM 5-AzaC or left untreated, and were seeded at a density of 1.0×10^4 cells per well in a 96W1E⁺ PET array chip (Applied BioPhysics Inc.) in appropriate culture medium with or without 5 µM 5-AzaC. An alternating current (~1 µA, 32 kHz) was applied to the electrodes to measure impedance (Ohms) and monitor proliferation in real-time for 20 h post-plating. Impedance in wells containing cells treated with 5-AzaC was compared to impedance in wells containing untreated control cells. A decrease in impedance was indicative of a decrease in cell proliferation.

For invasion assays, 96W1E⁺ PET array chips were coated with 25µg/ml bovine plasma fibronectin (Life Technologies) for one hour, rinsed and seeded with bovine lung microvessel endothelial cells (BLMVEC; VEC Technologies, Rensselaer, NY) at a density of 1.0×10^5 cells per well. Upon confluency, an equal number of tumoral cells were gently added to each well. An alternating current (~1 µA, 4 kHz) was applied to the electrodes to measure impedance (Ohms) and monitor extravasation of the tumoral cells through the endothelial cell monolayer in real-time for 20 h post-plating. Impedance in wells containing tumoral cells was compared to impedance in wells containing healthy, non-tumoral control cells, or impedance in wells containing tumoral cells pre-treated with 5 µM 5-AzaC. Decreased impedance signified disruption of the endothelial cell monolayer by invasive cancer cells.

Quantitative reverse-transcription (RT)-PCR

RNA was extracted from cells using an RNeasy Mini Plus kit (Qiagen, Valencia, CA) and cDNA was synthesized using M-MLV Reverse Transcriptase (USB, Cleveland, OH), according to the manufacturer's protocol. All primers were designed using Primer3 software, based on sequences found in the National Center of Biotechnology Information (NCBI) GenBank. Primers each spanned an intronic region to prevent amplification of genomic DNA.

Quantitative RT-PCR (qRT-PCR) assays, using SYBR green technology, was carried out on an Applied Biosystems 7500 Fast Real Time PCR instrument (Applied Biosystems, Carlsbad, CA). Primers for 11 genes were designed to study their relative expression levels after 5-AzaC (5 µM) treatment. Reference genes were selected from panels validated by other groups for dog [27], cat [28], and human mammary samples [29].

Four dog reference genes, four cat reference genes and 13 human reference genes were tested on healthy and tumoral cells from each species. Only genes with Ct values < 28 and with a difference < 2 between healthy and tumoral cells, were included as reference genes. An overview of all qRT-PCR primers can be found in Table 1. All samples (n=3) were run in triplicate and the comparative C_t method ($2^{-\Delta\Delta C_t}$) was used to quantify gene expression levels where $\Delta\Delta C_t = \Delta C_t(\text{sample}) - \Delta C_t(\text{references})$. Calculations were performed using the 7500 Fast software associated with the real time PCR thermal cycler.

Bisulfite sequencing

Genomic DNA was extracted from cells using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Bisulfite treatment of DNA was carried out with the MethylCode Bisulfite Conversion Kit, according to manufacturer's instructions (Invitrogen Life Technologies, Grand Island, NY). CpG islands upstream of the *PGP9.5* gene were identified based on sequence information in the UCSC genome browser (<https://genome.ucsc.edu>). Primers to amplify an upstream CpG island were designed with MethPrimer software (<http://www.urogene.org/methprimer>) which amplified a 212 base pair region containing 16 CpGs. Traditional PCR using Taq DNA Polymerase (Invitrogen Life Technologies) was performed to amplify the region of interest and a portion of the PCR products were run on a 1.5% agarose gel containing GelRed intercalating dye (Thermo Fisher Scientific, Waltham, MA) at 97 V for 1 h. Remaining PCR products were purified using the PCR Purification Kit (Qiagen, Valencia, CA) and cloned into DH5 α competent *E. Coli* using the pGEM T-easy vector (Promega, Madison, WI). Transformed *E. Coli* were plated on Luria-Bertani (LB) agar (Thermo Fisher Life Technologies, Grand Island, NY) containing 100 $\mu\text{g/mL}$ ampicillin and incubated for 16

h at 37°C. White colonies were picked and used to inoculate 4 mL LB broth with ampicillin. After 16 h incubation at 37°C, cultures were centrifuged at 400 x g for 10 min and 4°C to pellet bacteria. Plasmid DNA was collected using the QIAprep Spin Mini Prep Kit (Qiagen, Valencia, CA) and a portion was digested with EcoR1 (NE Biolabs, Ipswich, MA) and run on a 1.5% agarose gel containing GelRed intercalating dye to assess insert length. From each treatment, five plasmid DNA samples containing inserts of the predicted length were sequenced at the Cornell University Bioresource Center. Sequences were aligned using Genious software and methylation status of the 16 CpGs was assessed.

Cell viability assays

To evaluate the cytotoxicity of 5-AzaC on both normal and tumoral mammary cell lines, cells were seeded at 10,000 per well in 96 well microplates. At 90% confluency, low (5 µM) or high (50 µM) doses of 5-AzaC were added to triplicate wells. After 48 h of culture, an MTT *in vitro* toxicology assay (Sigma Aldrich, Saint Louis, MO) was carried out, according to manufacturer's instructions, and absorbance was measured at 570 nm on a Multiskan EX plate reader (Thermo Fisher Scientific, Waltham MA). Optical densities of wells treated with 5-AzaC were compared to those of untreated wells to determine cell viability.

Mitotracker C staining

Cells for Mitotracker C staining were plated in 24 well culture dishes fitted with 35 mm coverslips. After one day of culture, 50 µM 5-AzaC was added to appropriate wells. After 48 h of treatment, cells were washed twice with PBS and incubated with the mitochondria-specific red fluorescent probe MitoTracker Red CMXRos (Cell Signaling

Technology, Danvers, MA) at a final concentration of 100 nM in serum free-culture medium for 45 min at 37°C. Cells were then washed 3 times with PBS and fixed with ice cold 90% ethanol for 10 min at -20°C. Cells were washed 3 times with PBS and incubated with 0.5 µg/mL DAPI (BioLegend, San Diego, CA) for 5 min in PBS. After 3 washes with PBS, coverslips were carefully removed from culture wells and mounted on glass slides using aqueous mounted medium (Dako, Carpinteria, CA). Samples were examined with a Zeiss LSM confocal microscope (Oberkochen, Germany) and images were captured with an attached camera controlled by ZEN imaging software.

Immunocytochemistry (ICC) analyses

Cells for ICC were grown in 24 well culture dishes fitted with 35 mm coverslips. After one day of culture, 50 µM 5-AzaC was added to appropriate wells. After 48 h of treatment, cells were rinsed with PBS and fixed in 4% PFA for 10 min. Following 3 rinses with PBS, cells were permeabilized using PBS + 1% Triton-X 100 + 1% BSA for 30 min at RT. Primary rabbit anti-active caspase-3 antibody (Abcam, Cambridge, MA) or rabbit IgG, each diluted 1:100 in PBS, was added to the wells and incubated overnight at 4°C. Wells were then rinsed 3 times with PBS, and HRP-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA) diluted 1:100 in PBS was added. After 30 min at RT, cells were washed 3 times with PBS and AEC solution (Invitrogen Life Technologies, Grand Island, NY) was added for 15 min. Cells were rinsed with PBS, counterstained with Gill's Hematoxylin (Thermo Fisher Scientific, Waltham, MA) and washed with tap water. Coverslips were gently removed from wells and mounted on slides using aqueous mounted medium (Dako, Carpinteria, CA). Images were captured with a digital camera mounted on an Olympus BX51 light microscope

(Center Valley, PA). To determine percentage of cells positive for active caspase-3, cells in three fields each containing at least 300 cells, were counted and classified as either positive or negative based on presence or absence of red staining.

Statistical analyses

All experiments were repeated at least three times. Results are expressed as the mean \pm STDEV from three independent experiments, with the exception of the (ECIS) data, which show the mean \pm STDEV of endpoint impedance from 3 replicate wells of one experiment, representing statistical trends calculated from 3 independent assays. Data were analyzed by the Student's T-test and p values < 0.05 were considered statistically significant. Single and double asterisks indicate $p < 0.05$ and $p < 0.01$, respectively.

Results

Characterization of in vitro tumorigenicity of canine and feline mammary carcinoma cell lines and primary patient-derived mammary tumor cells.

To characterize the *in vitro* tumorigenicity of the canine and feline tumoral cell lines and primary tumor cells used in this study, soft agar and invasion/extravasation Electric Cell-substrate Impedance Sensing (ECIS) assays were performed. The soft agar assays, used to assess anchorage-independent growth, showed that significantly more spheres were formed by the REM134 and K12-72.1 cell lines than by the CMT12 and Cat-MT cell lines, respectively, and that these spheres had a tendency to a larger size (Figure 1A). The ECIS assays, used to model tumor extravasation and assess the metastatic potential of these cell lines did not show a statistically significant decrease in impedance from confluent monolayers of bovine lung microvessel endothelial cells (BLMVEC) co-

cultured with any of the tumoral cell lines, when compared to BLMVEC co-cultured with healthy control cell lines (Figure 1B).

The tissue samples that were used to isolate primary canine and feline mammary adenocarcinoma-derived cells (CMADC and FMADC, respectively) were evaluated on histopathology based on cell type involved, tubule formation, mitotic index and nuclear variation, and scored as a grade 2 complex mammary carcinoma (canine tissue) and a grade 2 simple mammary carcinoma (feline tissue) (Figure 2A). When using CMADC and FMADC in soft agar assays to assess their anchorage-independent growth capacities, however, no clear spheres were formed, not even after 40 days of culture (data not shown). In contrast, CMAD and FMAD did show invasive potential using ECIS, since co-culturing BLMVEC with these tumor cells resulted in a statistically significant decreased impedance compared to co-culturing with healthy control cell lines (Figure 2B).

Treatment of breast/mammary cancer cell lines and primary tumor cells with a low dose of 5-AzaC suppresses both anchorage-dependent and -independent tumoral growth, as well as tumor invasion activity.

The effects of the DNA methylation inhibitor 5-azacytidine (5-AzaC) on tumor proliferation (anchorage-dependent growth), *in vitro* tumorigenicity (anchorage-independent growth) and tumor invasion potential of the canine, feline and human tumoral cell lines were evaluated using a low dose of 5 μ M 5-AzaC. Briefly, although a significant reduction in viability of the cell lines MCF10CA1a, CMEC and CMT12 was observed after treatment with this low dose of 5 μ M 5-AzaC, the overall tendency was this low dose did not negatively affect viability of the tumoral cells as determined by

MTT assays (Suppl. Figure 1). Indeed, since the absolute variation between the three replicates of MCF10CA1a, CMEC and CMT12 was extremely small (Suppl. Figure 1), the biological relevance of the statistically significant difference in these cell lines could be questioned. To evaluate tumor proliferation, cells were treated with 5-AzaC or left untreated (control) for 2 days, after which each cell line was added to wells of an ECIS array slide at low density. Increases in impedance (a direct measure of the surface area of the well covered by adherent cells) were recorded. As seen in Figure 3A, a significantly lower impedance was observed in most of the tumoral cells lines treated with 5-AzaC when compared to untreated controls, indicating that 5-AzaC treatment of tumoral cells from all three species correlated with a decrease in proliferation. The exceptions were the CAT-MT cell line, which showed a decrease in impedance when treated with 5-AzaC that did not reach significance, the MCF7 cell line, which showed no difference in impedance upon treatment, and the CMADC cells, which showed a significantly higher impedance upon treatment (Figure 3A). When looking at the effects of 5-AzaC treatment in soft agar assays, a reduced anchorage-independent growth capacity of all tumoral cell lines was found as shown by a significantly reduced number of spheres, as well as a reduced size of these spheres that reached significance for MCF10CA1a, MCF7 and CMT12 (Figure 3B). The effects of 5-AzaC treatment on the anchorage-independent growth of the primary tumor cells CMAD and FMADC was not assessed since these cells do not form spheres in soft agar, as previously determined (data not shown).

Finally, the effects of 5-AzaC treatment on tumor invasiveness was evaluated using the ECIS system. For these assays, only those cell lines/primary cells were used that showed invasive potential based on our experiments using ECIS (Figure 2B) or previous

literature (Table 2). When MCF10CA1a, CMADC and FMADC or were added to a confluent BLMVEC monolayer, they disrupted (invaded) the endothelial cells as visualized by a rapid and sustained decrease in impedance as the endothelial cells were displaced from the surface of culture wells (Figure 4). In contrast, when these cells were treated with 5-AzaC they no longer induced a decrease in impedance as compared to the untreated cells, indicating that 5-AzaC treatment of these human tumoral cells significantly suppressed their metastatic potential (Figure 4).

Treatment with a low dose of 5-AzaC affects gene expression with both inter- and intra-species variability and the modified gene expression is caused by DNA methylation.

To evaluate the effects of 5-AzaC treatment on gene expression, 11 genes were selected that were shown previously to be upregulated upon 5-AzaC treatment in human breast cancer cell lines [30-32]. An initial screen revealed that some genes were downregulated whereas others were upregulated after 5-AzaC treatment (Table 3). Interestingly, no consistent pattern was observed in gene expression between tumoral cells from the 3 different species, and more importantly, not even between different tumoral cell lines from the same species. For example, the gene *DKK3* was upregulated in the human cell line MCF10CAa1 and the feline cell lines K12-72.1 and primary feline FMADC, but not in MCF7 or any of the canine cell lines/primary cells (Table 3). Another example is the gene *PGP9.5*, which was upregulated > 4-fold in CMADC, the canine primary tumor cells, but not in the canine cell lines CMT12 and REM134 (Table 3). Next, the increase in expression of those genes that were upregulated > 4-fold after 5-AzaC treatment was confirmed by repeating the qRT-PCR assays. The genes *FKBP6*, *SYK* and *PGP9.5* were significantly upregulated in all cell lines/primary cells tested

(Figure 5A). The gene *NTN4* was significantly upregulated in FMAD but did not reach significance in MCF10CA1a, and the gene *SFRP1* was upregulated in FMADC albeit also without significance (Figure 5A). Since 5-AzaC is known to function as a DNA methyltransferase (DNMT) inhibitor at low doses [33], bisulfite sequencing was used to confirm the DNA methylation-dependent action of this drug in our current study. To this end, the K12-72.1 cell line was used to evaluate the methylation status of CpG sites in the promoter region of the gene *PGP9.5*. This gene was chosen based on the fact that 5-AzaC treatment, at both 5 and 10 μ M, induced a significant and robust upregulation in this cell line (Suppl. Figure 1B). Sixteen CpG sites were examined and all 16 sites were found to be methylated in untreated K12-72.1 (Figure 5B). In contrast, cells treated with 5 or 10 μ M 5-AzaC only had between 12-15 and 1-14 CpG sites, respectively, methylated (Figure 5B). These results showed that at low concentrations 5-AzaC indeed causes demethylation of CpG sites in the promoter region of *PGP9.5* (Figure 5B) and that this is associated with increased expression of this gene (Suppl. Figure 1B).

Treatment with a high dose of 5-AzaC has a direct toxic effect on breast/mammary cancer cell lines and primary tumor cells, without affecting healthy mammary cells.

Aside from demethylating cellular DNA, 5-AzaC has been shown to cause DNA damage by inducing double-strand breaks and to induce apoptosis by mitochondrial membrane permeabilization and caspase activation in cancer cells like myeloma cells [34, 35]. To begin exploring these cytotoxic effects of 5-AzaC in breast/mammary cancer cells, we treated the canine, feline and human cancer cells with a high dose of 5-AzaC (50 μ M) and evaluated viability using MTT assays. At this concentration, a significant reduction in cell viability was observed in treated tumor cells as compared to untreated,

control cells (Figure 6A). Importantly, such an effect was not observed when normal health mammary cell lines were treated with the same dose of 5-AzaC, with the exception of a small, but significant, decrease in viability in the healthy canine mammary cell line CMEC (Figure 6A). These data show that this drug is selectively toxic towards tumor cells as compared to normal cells. To study the underlying mechanisms of this increased toxicity in more detail, we performed a staining with the MitoTracker Red CMXRos on untreated and 50 μ M 5-AzaC-treated tumor cells and observed a loss of dye accumulation in the treated tumoral cells, indicative of mitochondrial membrane permeabilization, when compared to control tumoral cells (Figure 6B). Moreover, 5-AzaC treatment activated caspases in mammary tumoral cells, as shown by an increased expression of active caspase-3 in treated versus untreated tumoral cells (Figure 7). In contrast, no such effects were observed when healthy mammary cells from all three species were treated with 50 μ M of 5-AzaC (Figure 6B and Figure 7), re-emphasizing the selective toxic effects of 5-AzaC on tumoral cells, at least *in vitro*.

Discussion

The present comparative study was initiated to evaluate the effects of the epigenetic modifier 5-AzaC on mammary tumorigenicity of canine and feline mammary cancer cells *in vitro*, and to compare these results with results obtained in human breast cancer cell lines. Our salient findings were that 5-AzaC at a low concentration (5 μ M) could reduce *in vitro* tumorigenicity and at a high dose (50 μ M) had a direct toxic effect for tumoral, but not healthy, mammary cells in all three species. The consistency of our results across the three species supports the value of naturally occurring mammary cancer in dogs and

cats as a valuable translational model for human breast cancer, and provides the *in vitro* rationale for using DNA methyltransferase (DNMT) inhibitors, like 5-Aza, as a potential treatment option in veterinary oncology.

For our *in vitro* tumorigenicity studies, we used a combination of assays to evaluate the anchorage-dependent and -independent growth, and invasive potential, of tumoral cells in the presence and absence of 5 μ M 5-AzaC. For the anchorage-dependent assays, tumor cells were plated in 96W1E⁺ PET plates and cell growth, also defined as proliferation, was measured over time using Electric Cell-substrate Impedance Sensing (ECIS). Anchorage dependence is a phenomenon that has been defined as an increase in proliferation when cells are allowed to attach to a solid surface and is therefore relevant to malignant transformation and tumorigenicity [36,37]. All tumoral cell lines showed a significant reduced proliferation upon treatment with 5-AzaC with exception of the feline CAT-MT cell line (reduction but not significant), the human MCF7 cell line (no effect) and the canine primary tumor cell CMADC (significantly increased expression). Despite this unexpected increase in proliferation of CMAD after 5-AzaC treatment, 5-AzaC could still inhibit the invasiveness of these cells as shown by the tumor invasion/extravasation ECIS assay [39, 40]. Interestingly, a study evaluating the effects of 5-AzaC on proliferation and *in vitro* invasion of pancreatic adenocarcinomas also found contradictory and adverse effects of 5-AzaC in some, but not all, pancreatic cell lines [41]. More specifically, they found that whereas 5-AzaC could inhibit the proliferation of all five pancreatic cancer cell lines, a significant increase, instead of the expected decrease, in *in vitro* invasive potential was noted in four out of the five cell lines after treatment with 5Aza [41]. Those data combined with our results indicate that the use of

methylation inhibitors to reduce tumorigenicity should be carefully evaluated, ideally using the primary tumor cells isolated from the patient's tumor to ensure that these drugs will not results in adverse effects when used clinically in that patient.

To study anchorage-independent growth, soft agar assays were used. These functional *in vitro* assays are standard for modeling *in vitro* tumorigenicity, at least for human cancer cells, and correlate fairly well with *in vivo* carcinogenesis [38]. Since soft agar assays are not routinely used to evaluate anchorage-independent growth of feline and canine cancer cells, we decided to first characterize the sphere formation of the tumoral cell lines and primary cells used in this study. All cell lines (CMT12, REM134, CAT-MT and K12-72.1) were capable of forming tumor spheres, however, and to our surprise, the primary cells CMAD and FMAD were unable to form spheres in soft agar. A potential explanation for this lack of sphere formation could be the specific requirements of these patient-derived primary tumor cells and consequently, optimization of the soft agar concentrations, seeding density, culture media (increasing serum, adding hormones like EGF or IGF-1) and environmental conditions (oxygen, humidity) might be necessary for these primary cells to be able to form spheres in the soft agar assay.

In addition to studying the effects of 5-AzaC on *in vitro* tumorigenicity of breast/mammary cancer cells in all three species, we evaluated the expression profiles of genes that were shown previously to be upregulated upon 5-AzaC treatment in human breast cancer cell lines. Ten genes were shown to be upregulated in MCF7 cells [30] and one gene, *SYK*, was shown to be upregulated in six *SYK*-negative breast cancer cell lines [31-32]. When we evaluated the expression of those ten genes in 5-AzaC-treated MCF7 cells as a positive control, we only observed an upregulation in mRNA expression of

440 *SFRP1* and *NTN4*. This discrepancy might be explained by differences in the qRT-PCR
441 assay used to assess mRNA expression. The authors of the previous study used a TaqMan
442 approach and included only one reference gene, *GAPDH*, whereas we used SYBR green
443 and two reference genes, namely *GAPDH* and *HSPBC*. Another explanation could be the
444 treatment regiment: MCF7 cells were treated with an undisclosed amount of 5-AzaC for
445 3 days in the referenced study, instead of 5 μ M 5-AzaC for 2 days that was used in our
446 present study. Still, when looking at the effects of 5AzaC on expression of the same
447 genes but in the 3 different species, not only considerable inter-species variability was
448 observed but also substantial intra-species variability, at least in this limited set of 11
449 genes. The latter is in line with what has been reported in the referenced study related to
450 the 5-AzaC-treated MCF7 [30], where they found that the genes that were upregulated in
451 5-AzaC-treated MCF7 showed variable levels of gene expression in other breast cancer
452 cell lines, indicating that a variable level of DNA methylation of the same genes exists in
453 different cancer cell lines. Although not the focus of our present study, this variability
454 between different tumors from the same tissue, like the mammary gland, could
455 potentially reduce the usefulness of DNA methylation of specific genes as biomarkers.

456 Finally, we also used 5-AzaC at a high dose to evaluate its direct cytotoxic effects on
457 cancer cells, as previously described [34, 35]. Treatment of these tumor cells with 50 μ M
458 5-AzaC could significantly reduce viability of these cells, an effect caused by apoptosis
459 as determined by mitochondrial permeabilization and caspase-3 activation. Importantly,
460 treating healthy mammary cell lines with a high dose of 5-AzaC did not negatively affect
461 viability, indicating the selective toxicity of this drug towards tumoral cells, at least *in*
462 *vitro*. Confirming that apoptosis is the process by which a high dose of 5-AzaC reduces

viability in canine and feline mammary cancer cell lines (i) suggests that these cells behave like human mammary cancer cell lines, supporting the use of dogs and cats as models for human cancer and (ii) directs future drug studies toward exploring the power of combination (epigenetic) therapies to induce potent and directed killing of dog and cat tumor cells. Indeed, work with human breast cancer cell lines has shown that treatment with 5-AzaC in combination with overexpression of the tumor suppressor gene Inhibitor of growth family member 1 (*ING1*), a critical epigenetic regulator of cellular senescence, had a synergistic cytotoxic effect [42]. Although each of these compounds causes dysregulation of a distinct epigenetic pathway, they were shown to complement each other by ultimately directing target cells towards apoptosis.

Based on the *in vitro* findings in our current study, the next step will be to evaluate the effects of 5-AzaC in a mouse xenograft model of canine and feline mammary cancer. For human breast cancer cell lines, the anti-cancer effects of 5-AzaC, alone or in combination with other epigenetic drugs, have been evaluated in mouse xenografts [15,32]. In contrast, and to our knowledge, 5-AzaC has not been evaluated in canine and feline xenograft models of mammary cancer to date. Therefore, future experiments are planned to first establish and characterize canine and feline mammary tumor xenograft models with the primary CMADC and FMADC used in the present study, and then use these patient-derived xenograft (PDX) models to evaluate the efficacy and safety of 5-AzaC, a drug for which we could show promising anti-cancer effects *in vitro*.

Conclusions

Taken together, we believe the results of the present study verify the unique comparative value of dogs and cats as models for breast cancer research in humans. More specifically, we propose that evaluating anti-cancer drugs in these animals will not only yield benefits for humans, but may improve treatments for veterinary species as well.

List of Abbreviations

DNMT: DNA methyltransferase; 5-AzaC: 5-Azacytidine; HDAC: histone deacetylase; FDA: Food and Drug administration; MCF10A: human normal breast epithelial cell line; MCF10CA1a: human malignant breast carcinoma cell line; DMEM: Dulbecco's modified Eagle medium; EGF: epidermal growth factor; ER: oestrogen receptor; MCF7: human breast adenocarcinoma cell line; FMEC: feline normal mammary epithelial cell line; K12-72.1: feline mammary adenocarcinoma cell line; CAT-MT: feline mammary carcinoma cell line; FBS: fetal bovine serum; CMEC: canine normal mammary epithelial cell line; REM134: canine mammary carcinoma cell line; CMT12: canine mammary carcinoma cell line; ECIS: Electric Cell-substrate Impedance Sensing; BLMVEC: bovine microvessel lung endothelial cells; qRT-PCR: quantitative reverse-transcription PCR; *DFNA5*: non-syndromic hearing impairment protein 5; *SFRP1*: secreted frizzled-related protein 1; *NTN4*: netrin 4; SYK: spleen tyrosine kinase; *FKBP6*: FK506 binding protein 6; *LOXL4*: Lysyl oxidase-like 4; *PON1*: paraoxonase 1; *TRIM50*: tripartite motif-containing 50; *OSPBL3*: oxysterol-binding protein 3; *DKK3*: dickkopf-related protein 3; *PGP9.5*: ubiquitin carboxy-terminal hydrolase L1; *HSPBC*: heat shock protein 1; *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase; *HPRT*: hypoxanthine guanine phosphoribosyl transferase; *UBI*: polyubiquitin; *RPL30*: Ribosomal Protein L30;

YWHAZ: 14-3-3 protein zeta; *ICC*: immunocytochemistry; *HRP*: horseradish peroxidase; PBS: phosphate buffered saline; BSA: bovine serum albumin; TBS: Tris buffered saline; PFA: 4% paraformaldehyde; *AEC*: 3-amino-9-ethylcarbazole; CMADC: canine mammary adenocarcinoma-derived cells; FMADC: feline mammary adenocarcinoma-derived cells; ECM: extracellular matrix; NIH-3T3: murine fibroblast cell line; EC: endothelial cells; ING1: inhibitor of growth family member 1; PDX: patient-derived xenograft.

Competing interests

The authors declare they have no competing interests.

Authors' Contributions

RH carried out all laboratory procedures, was involved in conception and design, and manuscript writing; TC provided expertise and technical assistance with the Electric Cell-substrate Impedance (ECIS) assays; DA provided canine and feline mammary cancer cell lines and SC provided the human cell lines; DA, SC and GVdW were involved in conception and design; GVdW was involved in data analyses and manuscript writing. All authors read and approved the final manuscript.

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Figure Captions

Fig 1 Characterization of *in vitro* tumorigenicity of canine and feline mammary cell lines. (A) Representative phase contrast images of tumorspheres formed by canine and feline mammary cell lines in anchorage-independence (soft agar) assays, and quantification of sphere number and size determined from soft agar assays. $n=3$, *: $P < 0.05$, scale bars = 50 μm (B) Assessment of invasive potential of canine and feline mammary cell lines measured by invasion/extravasation Electric Cell-substrate Impedance Sensing (ECIS) assays. Representative phase contrast images of healthy canine and feline mammary cell lines, and non-invasive canine and feline mammary cell lines on top of endothelial cell monolayer in ECIS assay plate are shown. $n = 3$, scale bars = 50 μm

Fig 2 Characterization of primary mammary tumor cells cultured from canine and feline mammary adenocarcinomas. (A) Images of formalin-fixed, paraffin-embedded tumor tissues stained with hematoxylin and eosin. scale bars = 50 μm (B) Assessment of invasive potential of canine and feline adenocarcinoma-derived cells (CMADC and FMADC, respectively) measured by invasion/extravasation Electric Cell-substrate Impedance Sensing (ECIS) assays. Representative phase contrast images of healthy canine and feline mammary cell lines on top of endothelial cell monolayer in ECIS assay plates (upper images), and invasive CMADC and FMADC incorporated into endothelial cell monolayer (lower images) are shown. $n = 3$, scale bars = 50 μm

Fig 3 Effects of low dose 5-AzaC on anchorage-dependent and –independent growth of breast/mammary cancer cell lines and primary tumor cells. (A) Anchorage-dependent growth as measured by proliferation using Electric Cell-substrate Impedance

Sensing (ECIS) of canine, feline and human tumoral mammary cells lines/primary cells treated with 5 μ M 5-AzaC or left untreated. **(B)** Anchorage-independent growth as measured by soft agar assays of canine, feline and human tumoral mammary cells lines treated with 5 μ M 5-AzaC or left untreated. Number and size of spheres were determined. n=3, *: P < 0.05, **: P < 0.01

Fig 4 Effects of low dose 5-AzaC on invasive potential of breast/mammary cancer cell lines and primary tumor cells. Invasive potential of MCF10CA1a, CMADC and FMADC treated with 5 μ M 5-AzaC or left untreated was measured by invasion/extravasation Electric Cell-substrate Impedance Sensing (ECIS) assays. Representative phase contrast images of untreated (invading) and 5-AzaC treated (non-invading) cells on endothelial cell monolayer in ECIS assay plates are shown. n= 3, scale bars = 50 μ m

Fig 5 Effects of low dose 5-AzaC on gene expression and methylation status in breast/mammary cancer cell lines and primary tumor cells. (A) Expression levels of the genes *PGP9.5*, *SFRP1*, *NTN4*, *FKBP6* and *SYK* in canine, feline and human tumoral mammary cells lines/primary cells treated with 5 μ M 5-AzaC as determined by qRT-PCR. Fold change from non-treated cells is shown. n=3, *: P < 0.05, **: P < 0.01. **(B)** Graphic representation of the methylation status of CpG islands in the upstream promoter region of the gene *PGP9.5* in untreated K12-72.1 cells, and K12-72.1 cells treated with 2 concentrations (5 and 10 μ M) of 5-AzaC using bisulfite sequencing. Each column represents a CpG site (16 total) and each row represents a genomic DNA clone (n=5). Blue blocks indicate a methylated CpG site, red blocks an unmethylated CpG site. A representative trace file showing a methylated cytosine (top panel), unaffected by

bisulfite treatment, and an unmethylated cytosine (bottom panel), converted into a uracil by bisulfite treatment and amplified as a thymine during PCR, at position 139 (black arrow) is shown.

Fig 6 Effects of high dose 5-AzaC on viability and mitochondrial membrane permeabilization in breast/mammary cancer cell lines and primary tumor cells. (A)

Viability of canine, feline and human tumoral mammary cells lines/primary cells treated with 50 μ M 5-AzaC as determined by MTT assays. Percent viable cells, compared to non-treated cells, set at 100%, are shown. $n=3$, *: $P < 0.05$, **: $P < 0.01$. **(B)** Representative confocal images of canine, feline and human tumoral mammary cells lines/primary cells treated with 50 μ M 5-AzaC, or left untreated, and stained with MitoTracker Red are shown. Scale bars = 10 μ m.

Fig 7 Effects of high dose 5-AzaC on caspase activation in breast/mammary cancer cell lines and primary tumor cells. Representative bright field images of canine, feline and human tumoral mammary cells lines/primary cells treated with 50 μ M 5-AzaC, or left untreated, and stained with anti-active caspase-3 antibodies are shown. Numbers show percentage of cells positive for anti-active caspase-3. Scale bars = 20 μ m.

Supplementary Figure 1.

(A). Viability of canine, feline and human tumoral mammary cells lines/primary cells treated with 5 μ M 5-AzaC as determined by MTT assays. Percent viable cells, compared to non-treated cells, set at 100%, are shown. $n=3$, *: $P < 0.05$. **(B).** Expression levels of the gene *PGP9.5* in the feline cell line K12-72.1 treated with 5 and 10 μ M 5-AzaC as determined by qRT-PCR. Fold changes from non-treated cells is shown. $n=3$, *: $P < 0.05$.

Table 1. Primers used for qRT-PCR

| A. Primers for genes reported to be affected by 5-AzaC* | | | | |
|--|---------------------|-------------------------------|--|--|
| Gene | Abbreviation | Species | Forward Primer (5'-3') | Reverse Primer (5'-3') |
| Non-syndromic hearing impairment protein 5 | <i>DFNA5</i> | human, canine, feline | AGCCACAACAGACAGCTTTG | ACTGGTTCAGGACCATGAG |
| Secreted frizzled-related protein 1 | <i>SFRP1</i> | human, canine, feline | TGTCCCAAGAAGAAGAAGC | AAGTGGTGGCTGAGGTTGTC |
| Netrin 4 | <i>NTN4</i> | human, canine, feline | AAACTCTGGGCAGACACCAC | TAGGCAGCATTGCACTTGTC |
| Spleen tyrosine kinase | <i>SYK</i> | human, canine, feline | AAACTACTACAAGGCCAGACC | TCCAGACGTCACCTTGCTG |
| FK506 binding protein 6 | <i>FKBP6</i> | human canine feline | TGACTTCCTGGACTGTGCTG GAATGCTAAGGCCCTCTTCC TTGAGCTGCTTGACTTCCTG | GTTCCGTAGCTGCCACTTTC TGAAGGGTTGTTCTTCTGG TAGTTGCCAACTCCCGTTC |
| Lysyl oxidase-like 4 | <i>LOXL4</i> | human canine feline | CCAGCTTCTGTCTGGAGGAC CGCTTCTCAGCTGGAGTTTC GCTTTGAAACAGCCTTGACC | ATATCCACCCACTGGCAATC CAGACTGGGAGAGGCAGTTC ATACAGCGCACATTGTCCAG |
| Paraoxonase 1 | <i>PON1</i> | human canine feline | AACCATCCAGATGCCAAGTC CAGAGGTGATCCGAATCCAG TATTGTTGCTGTGGGACCTG | AAAGTGCTCAGGTCCCACAG ACAGAGGCCACGGTACTTCC CCATCTGCCATCACTTGAAC |
| Tripartite motif-containing 50 | <i>TRIM50</i> | human canine feline | AACAGTTCGGCAATGAGGAC GCTAGCGGCTCTCATCTCTG CAGGCTGACATCAAGCTGAC | GCTTGATGTCAGCCTGGTG CGGGTCCTATTGTTCAACAG TTGGAGAGTTCCAGGAGTGG |
| Oxysterol-binding protein 3 | <i>OSBPL3</i> | human canine feline | CTATGCCGAAAGGCTACGAG TGCAGAATCCATGCTGAGTC GGAGTACAGCGAGCTTCTGG | CTGGTCTGGCCTAAATCGAG ACCAGAGCTCGGTTGTCATC GAACAGGATTGAAGGGCTTG |
| Dikkopf-related protein 3 | <i>DKK3</i> | human canine | TGTGACAACCAGAGGGACTG CTGTGCCTTCCAGAGAGGTC | CTAGCTCCCAGGTGATGAGG CAGGCTCTAACTCCCAGGTG |

| Ubiquitin carboxy-terminal hydrolase L1 | PGP9.5 | feline | CTGTGCCTTCCAGAGAGGTC | CAGGCTCTAACTCCCAGGTG |
|---|--------------|---------|-------------------------|------------------------|
| | | human | GAGATGCTGAACAAAGTGC | AGCCCAGAGACTCCTCTTCC |
| | | canine | GTGGTACCATCGGGCTTATC | TTCAGGACTGACCCATCCTC |
| | | feline | CAGTGGCCAATAACCAGGAC | GGTGACAGCTTCTCCGTTTC |
| B. Primers for reference genes | | | | |
| Gene | Abbreviation | Species | Forward Primer (5'-3') | Reverse Primer (5'-3') |
| Heat shock protein family B member 1 | HSPB1 | human | GGCATGACCAAAGCTGATCTC | ACCAAAGTGCCAATCATGG |
| Glyceraldehyde 3-phosphate dehydrogenase | GAPDH | human | GACAGTCAGCCGCATCTTCT | TTAAAAGCAGCCCTGGTGAC |
| Hypoxanthine phosphoribosyl transferase 1 | HPRT | canine | TGCTCGAGATGTGATGAAGG | TCCCCTGTTGACTGGTCATT |
| Polybiquitin | UBI | canine | TCTTCGTGAAAACCCTGACC | CCTTCACATTCTCGATGGTG |
| Ribosomal protein L30 | RPL30 | feline | CCTCGGCAGATAAATTGGACTGC | TGATGGCCCTCTGGAATTTGAC |
| Tyrosine 3-mono oxygenase/tryptophan 5-monooxygenase activation protein, zeta | YWHAZ | feline | GAAGAGTCCTACAAAGACAGCAC | AATTTTCCCCTCCTTCTCCTGC |

* These genes have been previously shown to be upregulated upon 5-AzaC treatment in human breast cancer cell lines [30-32].

Table 2. The invasive potential of the cell lines/primary tumor cells used in the present study.

| Species | Normal mammary/breast epithelial cell lines | Mammary/breast adenocarcinoma cell lines and cells | |
|---------|---|--|-------------------|
| | | Non-invasive | Invasive |
| Canine | CMEC | REM134 [25] [*] | CMADC |
| | | CMT12 [26] | |
| Feline | FMEC [22] | K12-72.1 [23] | FMADC |
| | | CAT-MT [24] | |
| Human | MCF10A [16] | MCF-7 [20] | MCF10CA1a [17,18] |

^{*} References for previously published cell lines are included in between brackets.

Table 3. Changes in gene expression after 5-AzaC treatment.

| Gene | Human cell lines | | Canine cell lines | | | Feline cell lines | | |
|---------------|------------------|-----------|-------------------|--------|-------|-------------------|----------|-------|
| | MCF7 | MCF10CA1a | CMT12 | REM134 | CMADC | CAT-MT | K12-72.1 | FMADC |
| <i>DFNA5</i> | - | ND | ND | ND | - | ND | ND | ND |
| <i>SFRP1</i> | + | + | - | + | + | - | + | ++ |
| <i>NTN4</i> | + | ++ | - | + | - | ND | ND | ++ |
| <i>FKBP6</i> | - | ++ | + | + | ++ | ND | + | + |
| <i>LOXL4</i> | - | ND | - | - | + | - | - | + |
| <i>PON1</i> | - | - | - | - | + | - | + | + |
| <i>TRIM50</i> | - | + | - | + | + | - | + | + |
| <i>OSPBL3</i> | ND | ND | - | - | - | - | - | + |
| <i>DKK3</i> | - | + | - | - | - | - | + | + |
| <i>PGP9.5</i> | - | ND | - | - | ++ | ND | ++ | + |
| <i>SYK</i> | - | + | + | + | - | - | - | + |

ND: not determined; -: no upregulation; +: < 4-fold upregulation; ++: > 4-fold upregulation

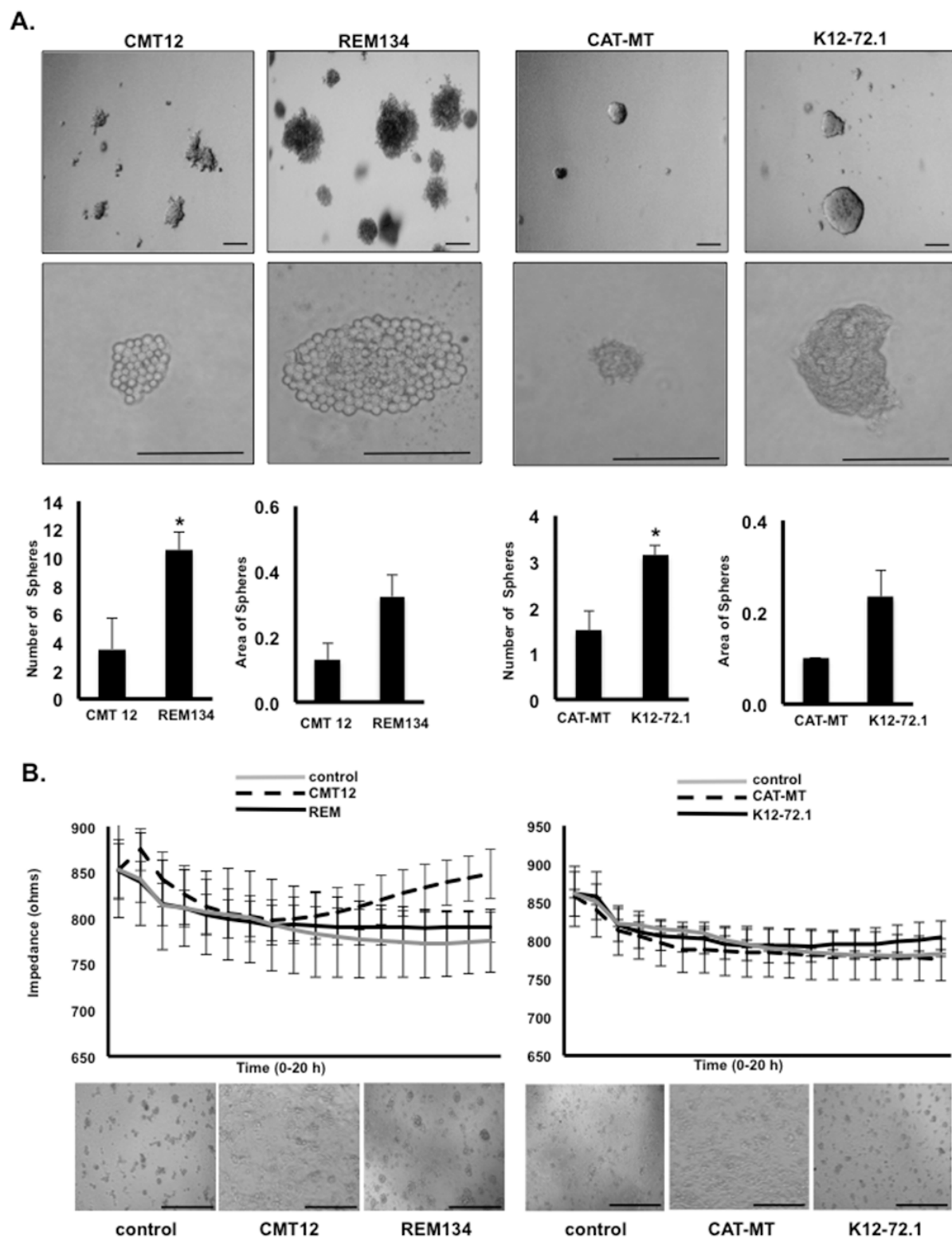
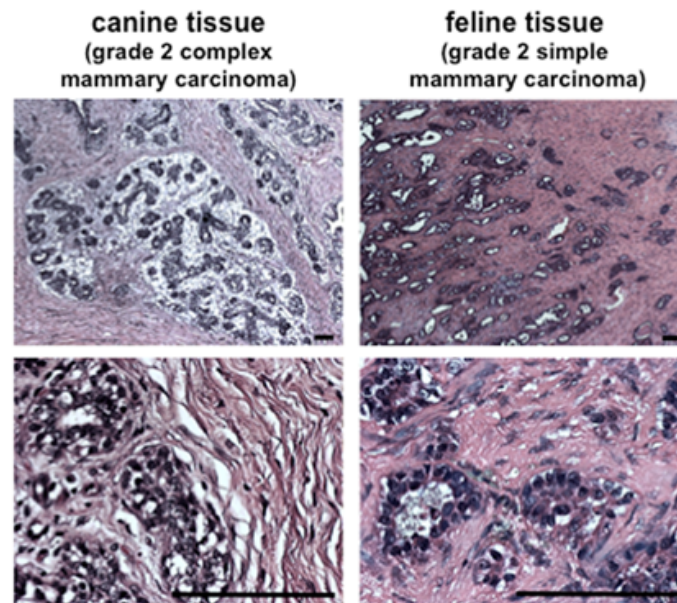


Figure 1. Harman et al.

A.



B.

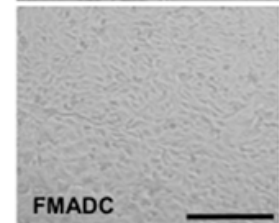
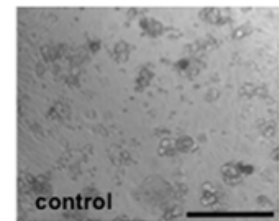
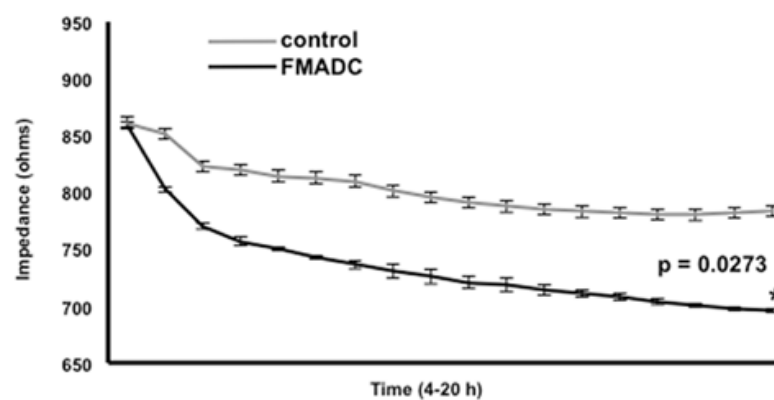
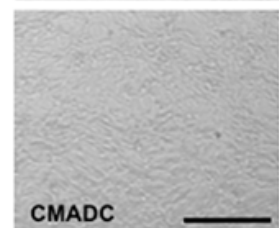
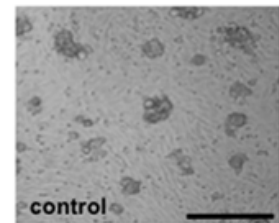
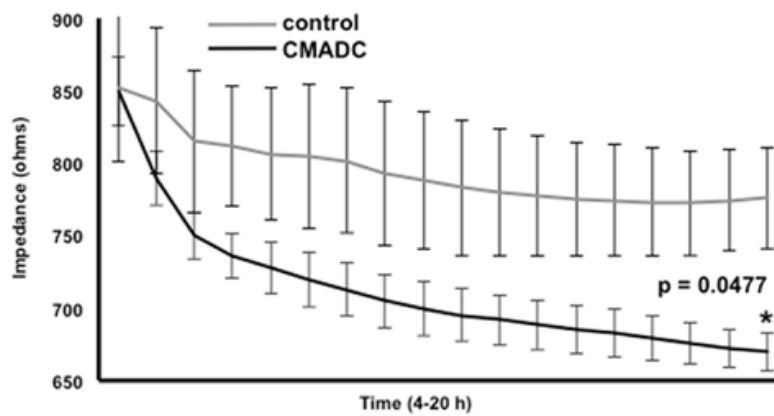


Figure 2. Harman et al.

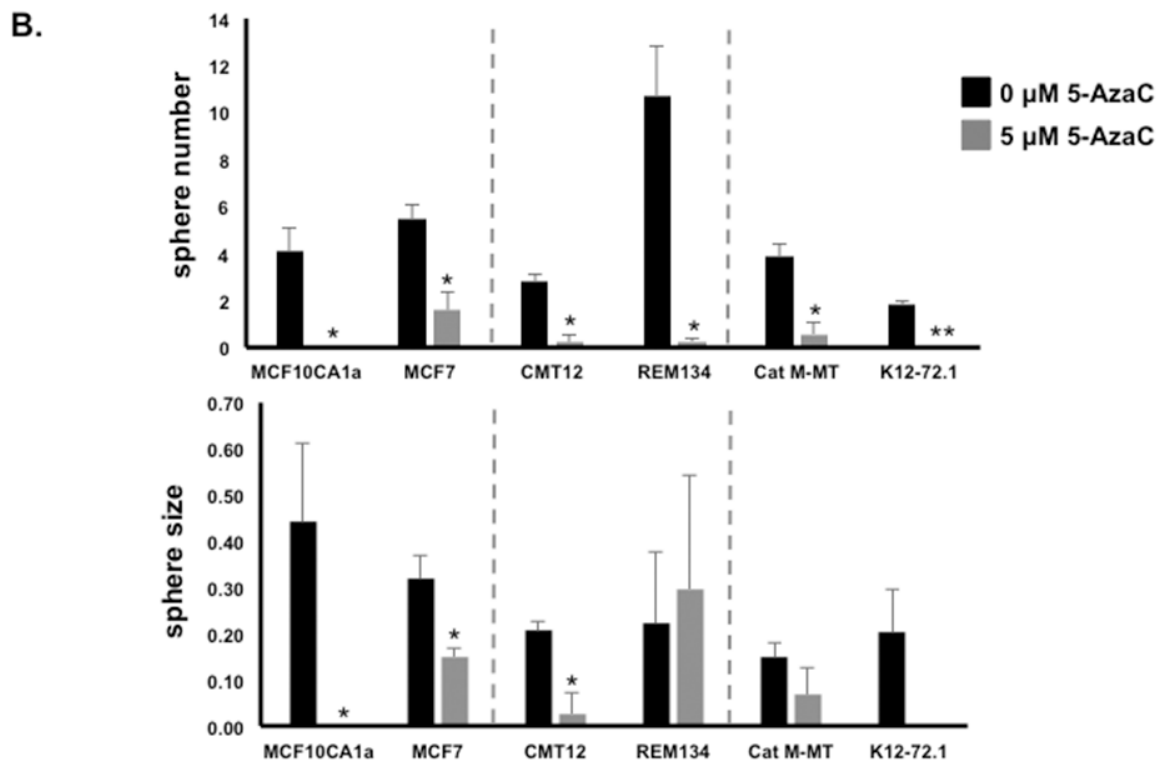
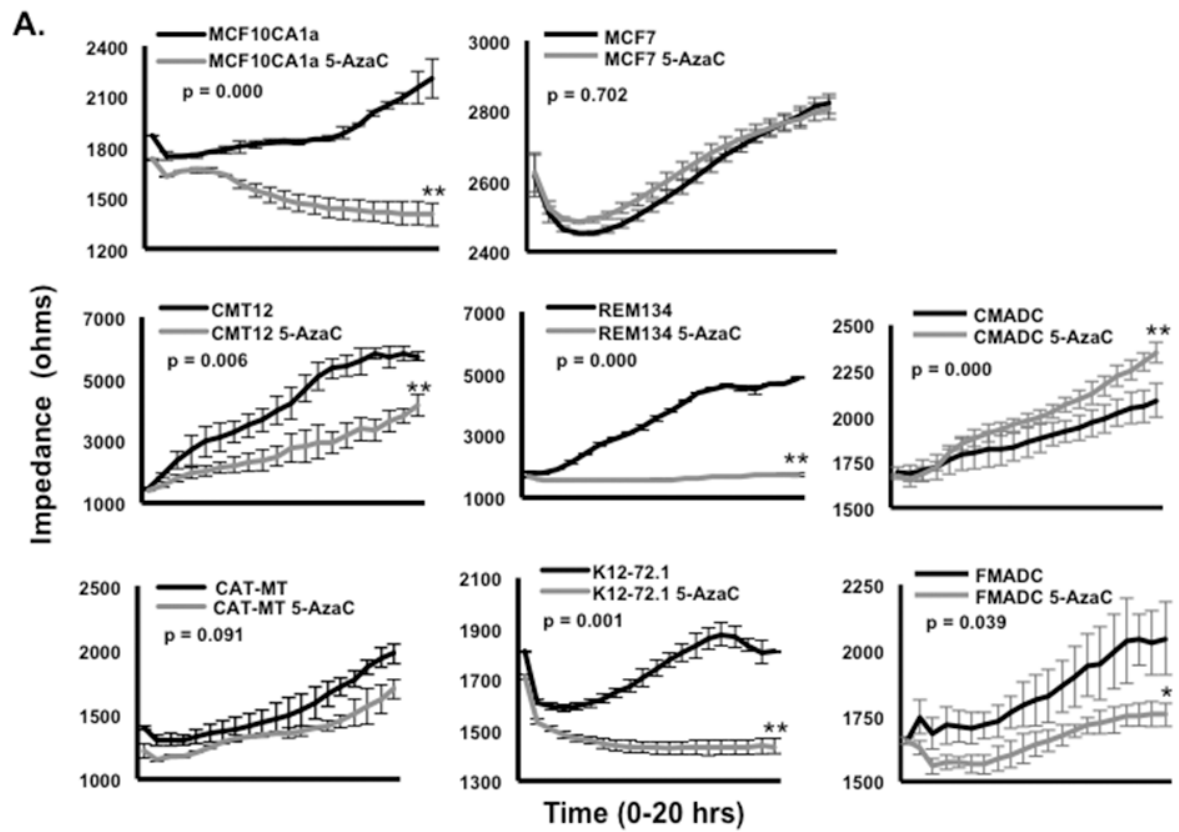


Figure 3. Harman et al.

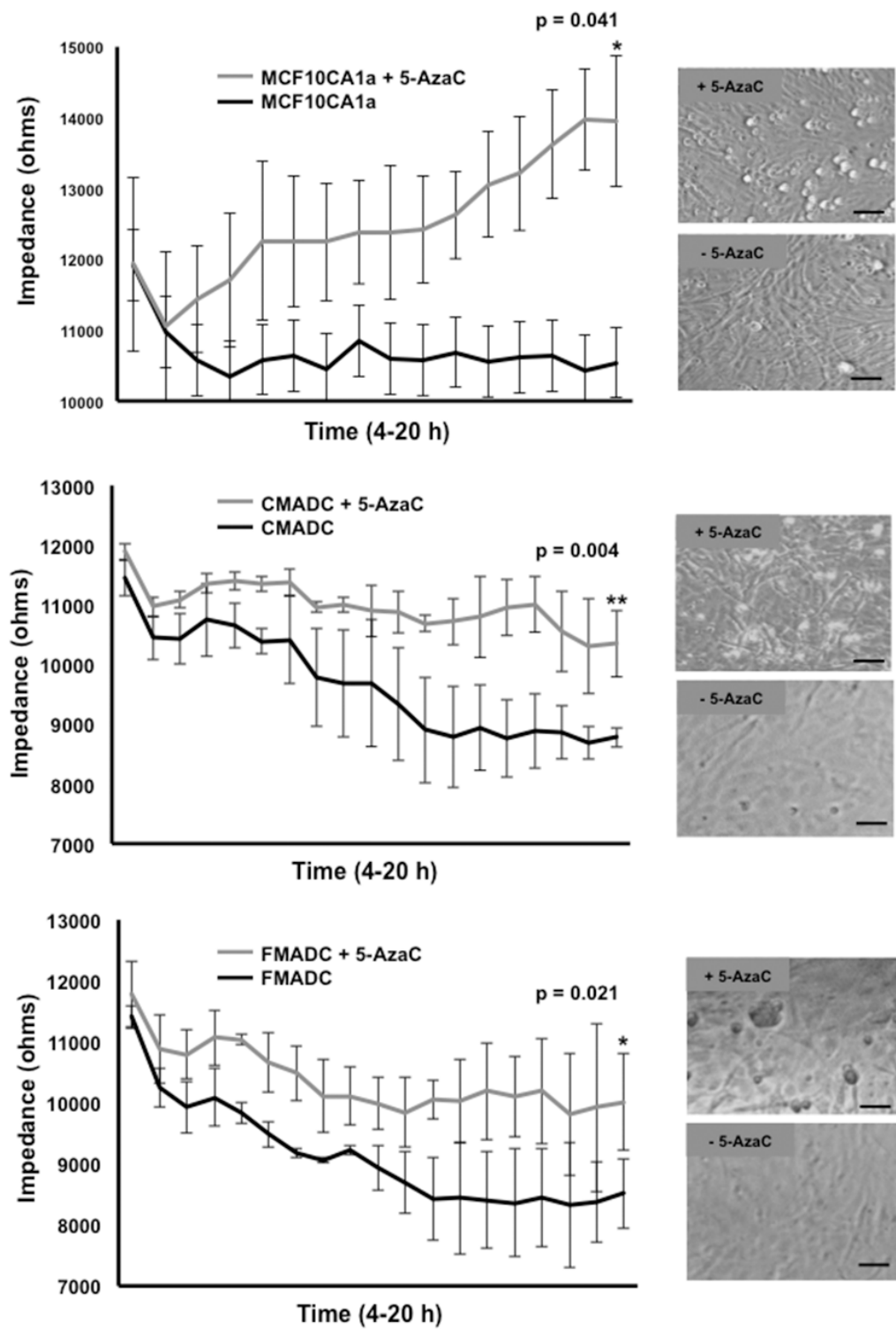


Figure 4. Harman et al.

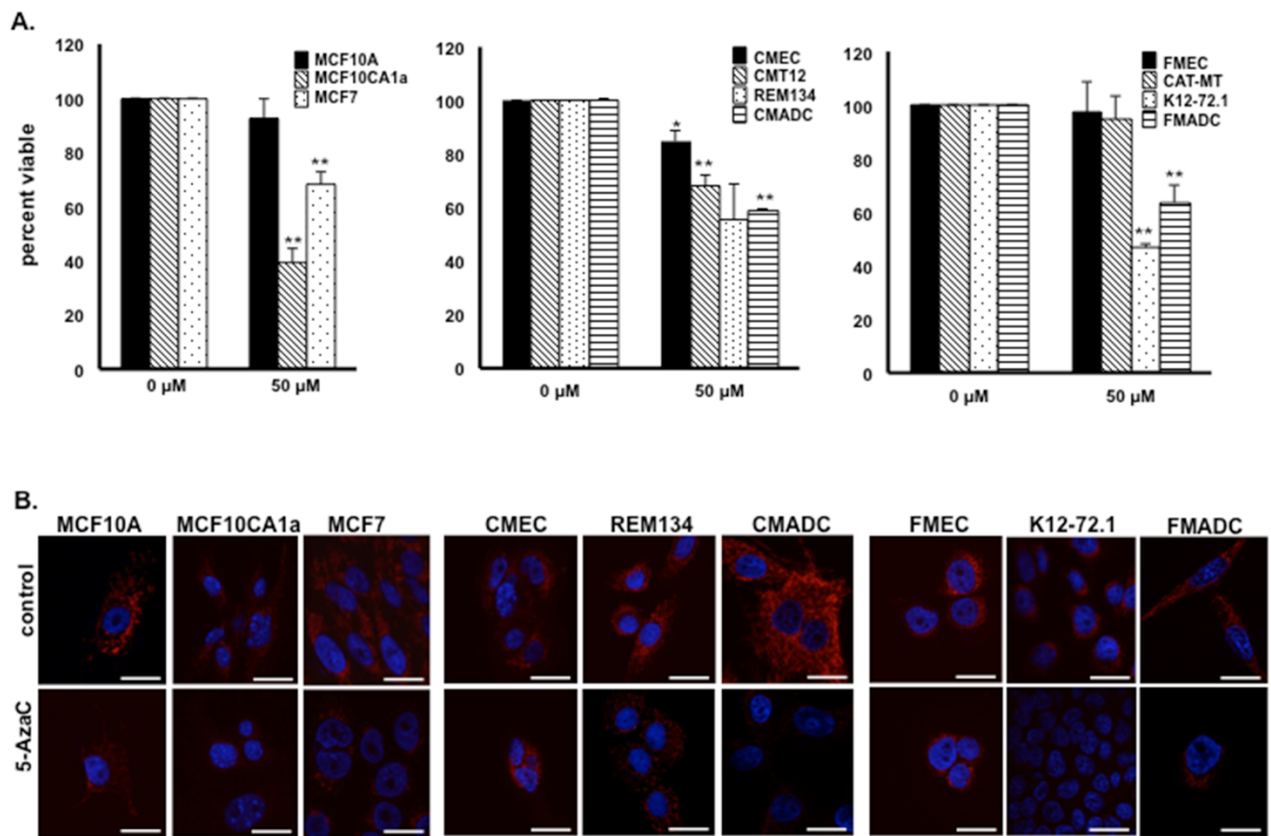


Figure 6. Harman et al.

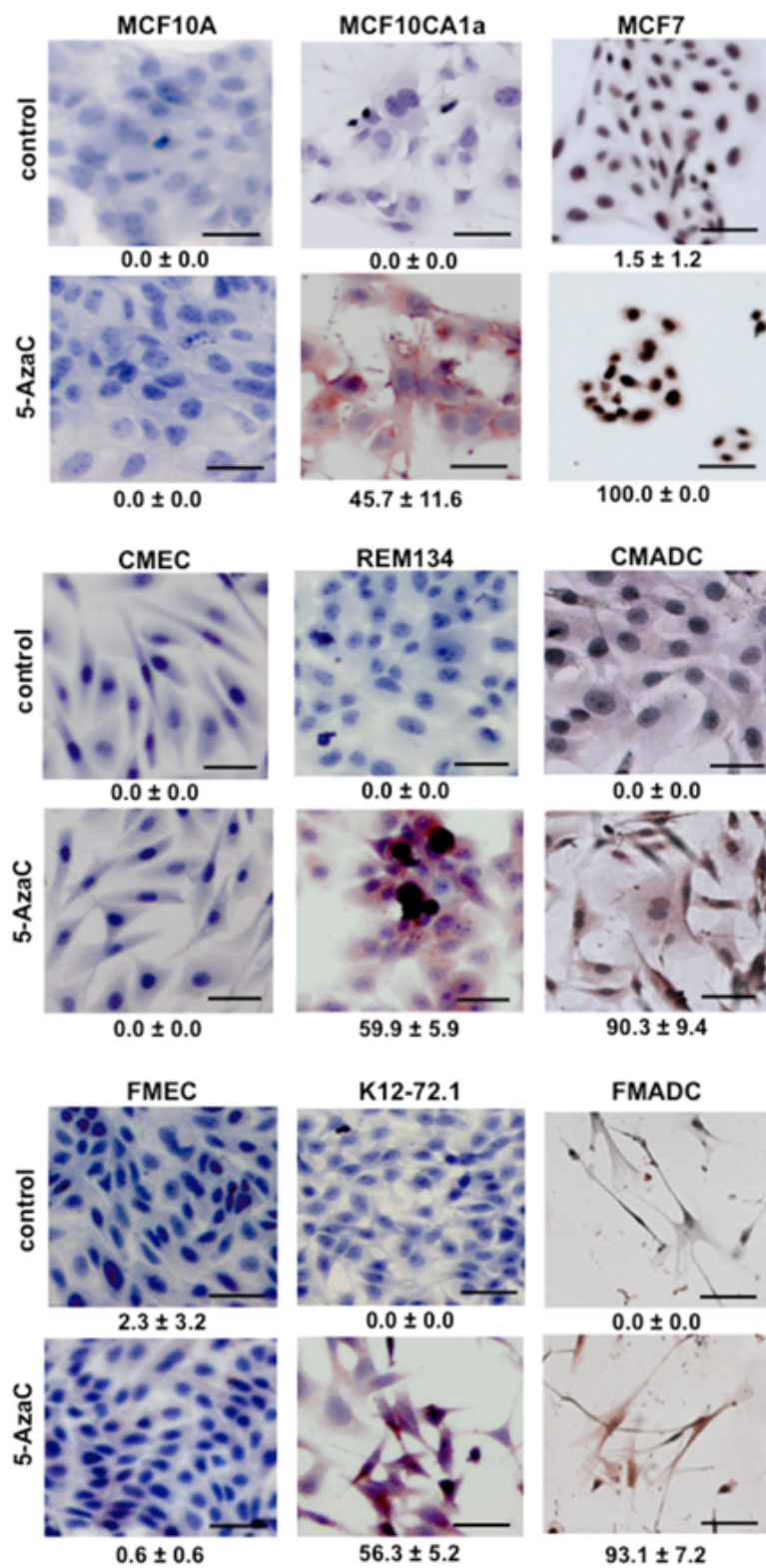
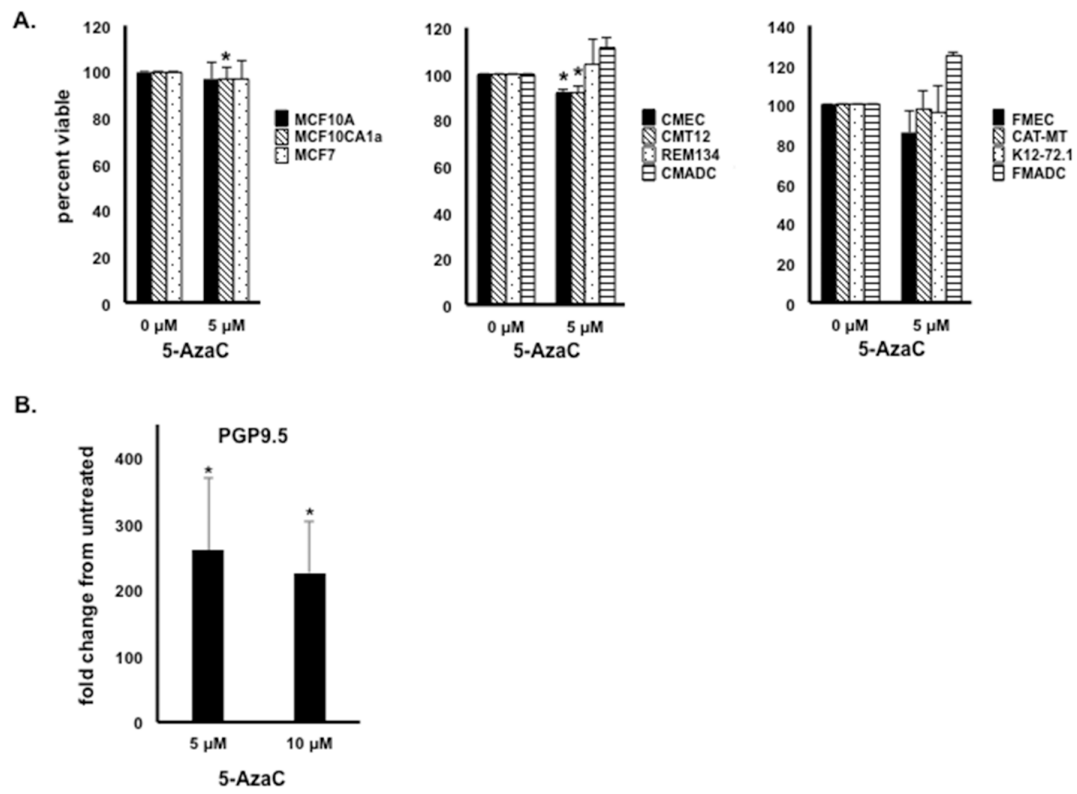


Figure 7. Harman et al.



Supplemental Figure 1. Harman et al.